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RESEARCH PAPER

Ultraviolet-B-induced oxidative stress and responses of the ascorbate–glutathione cycle in a marine macroalga *Ulva fasciata*

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Abstract

The regulation of the antioxidant defence system by ultraviolet-B (UV-B) was determined in a marine macroalga *Ulva fasciata* Delile exposed to low (0.5, 1 W m^{-2}), medium (2.5, 5 W m^{-2}), and high (10, 20 W m^{-2}) UV-B irradiance. UV-B ⩾2.5 W m^{-2} increased H_2O_2 contents that are positively correlated with lipid peroxidation and total peroxide contents. Inhibition of the UV-B-induced H_2O_2 increase by a specific $O_2^{\cdot-}$ scavenger, 1,2-dihydroxy-benzene-3,5-disulphonic acid, shows that $O_2^{\cdot-}$ is the primary source of H_2O_2. Superoxide dismutase activity was increased by UV-B with a peak at 2.5 W m^{-2}, which did not match the H_2O_2 pattern. Alleviation of UV-B-induced oxidative damage by a H_2O_2 scavenger, dimethylthiourea, and a free radical scavenger, sodium benzoate, which inhibited UV-B-induced H_2O_2 accumulation, suggests that oxidative damage caused by UV-B ⩾ 2.5 W m^{-2} is ascribed to accumulated H_2O_2. However, a decrease in growth rate and TTC reduction ability only at high UV-B doses indicates that the defence and repairing systems operate at low and medium UV-B doses. H_2O_2 not only can be excreted but can also be detoxified via the ascorbate–glutathione cycle. Increases in catalase, peroxidase, ascorbate peroxidase, and glutathione reductase activities and ascorbate (AsA) and glutathione pools, as well as AsA regeneration ability, function to keep the balance of cellular H_2O_2 under low UV-B doses. Dehydroascorbate reductase and monodehydroascorbate reductase are responsible for AsA regeneration under low and medium UV-B radiation, respectively. The appearance of oxidative damage in medium and high UV-B flux is attributable to a lower induction of the ascorbate–glutathione cycle as an antioxidant defence system. Overall, the availability of antioxidants and the induction of antioxidant enzyme activities for detoxifying reactive oxygen species (ROS) are regulated in *U. fasciata* against UV-B-induced oxidative stress, and experiments using ROS scavengers demonstrate that the antioxidant defence system is modulated by $O_2^{\cdot-}$ or H_2O_2.

Key words: Antioxidant, antioxidant enzyme, H_2O_2, macroalga, oxidative stress, reactive oxygen species, *Ulva fasciata*, UV-B.

Introduction

The stratospheric ozone layer is vital to life on Earth because it is the principal agent absorbing the ultraviolet radiation in the Earth's atmosphere. Since 1990, the depletion of the stratosphere ozone layer due to anthropogenic and natural destruction is leading to increasing levels of solar ultraviolet-B (UV-B: 280–320 nm) radiation reaching the Earth's surface, especially in Arctic, Antarctic, and temperate regions (Seckmeyer and McKenzie, 1992; Kerr and McElroy, 1993; Russell *et al*., 1996), where UV-B and UV-A (315–400 nm) are capable of penetrating the water column to an ecologically significant depth (Calkins and Thordardottir, 1982; Smith *et al*., 1992). Ambient UV-B irradiance at low latitudes is also high due to the high solar angle and a relatively low stratospheric ozone amount; the current UV-B flux at low latitudes is higher than any likely UV-B flux at higher latitudes even with further depletion of stratospheric ozone. High ultraviolet radiation can penetrate more deeply into the oligotrophic tropical water column because of the relatively low amount of dissolved and particulate organic matter (Baker *et al*., 1980). It would be expected that tropical seaweeds potentially encounter higher UV-B radiation than those occurring in temperate

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and Polar regions even under the normal ozone layer (Häder, 1993).

Exposure to UV-B leads to the generation of reactive oxygen species (ROS). In general, oxidative stress results from the disruption of cellular homeostasis of ROS production from the excitation of O_2 to form singlet oxygen (O_2^1) and the transfer of 1, 2 or 3 electrons to O_2 to form superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO^{\cdot}$), respectively (Halliwell and Gutteridge, 1989); the generating ROS leads to oxidative destruction of the cell components through oxidative damage of membrane lipids, nucleic acid, and protein (Davis, 1987; Wise and Naylor, 1987; McKersie and Leshem, 1994; Imlay and Linn, 1998). To counteract the toxicity of ROS, defence systems that scavenge cellular ROS have been developed in plants to cope with oxidative stress via the non-enzymatic and enzymatic systems (Noctor and Foyer, 1998; Asada, 1999). Antioxidants including water-soluble ascorbate (AsA) and glutathione and water-insoluble α-tocopherol and carotenoids have been considered to be the non-enzymatic agents for scavenging ROS (Noctor and Foyer, 1998; Smirnoff and Wheeler, 2000; Munné-Bosch and Alegre, 2002). In the enzymatic ROS-scavenging pathways, superoxide dimutase (SOD; EC 1.15.1.1) converts $O_2^{\cdot-}$ to H_2O_2 and then ascorbate peroxidase (APX; EC 1.11.1.11) and glutathione reductase (GR; EC 1.6.4.2) in the ascorbate–glutathione cycle (AGC) are responsible for H_2O_2 removal (Asada, 1999). It is known that APX utilizes AsA to reduce H_2O_2 by the oxidation of AsA to the monodehydroascorbate (MDHA) radical, which can be reduced by photosynthetic electron flow through ferredoxin or reduced to AsA by NAD(P)H-dependent monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) and MDHA would be spontaneously disproportionated to AsA and dehydroascorbate (DHA) (Mittler, 2002). DHA will then be reduced to generate AsA by dehydroascorbate reductase (DHAR; EC 1.8.5.1) using glutathione as an electron donor; the glutathione disulphide formed after glutathione oxidization is reduced to glutathione by GR utilizing reducing equivalents from NAD(P)H. Catalase (CAT; EC 1.11.1.6) (Willekens *et al*., 1997) and peroxidase (POX; EC 1.11.1.7) (Asada and Takahashi, 1987) are also involved in H_2O_2 removal. In addition to a role functioning as a toxic metabolite, H_2O_2 is also recognized as a signalling molecule that mediates the responses of plants to external biotic and abiotic stimuli (Vranova *et al*., 2002; Neil *et al*., 2002*a*, *b*).

The over-production of ROS and the induction of oxidative stress by UV-B radiation have been observed in microalgae such as *Chlorella vulgaris* (Malanga *et al*., 1997), cyanobacteria (Foyer *et al*., 1994; He *et al*., 2002), a diatom (Rijstenbil, 2002), *Euglena gracilis* (Tschiersch and Ohmann, 1993), and a symbiotic dinoflagellate (Lesser, 1996). An increase in the activities of ROS scavenging enzymes (such as SOD, APX, and CAT), and in the concentrations of antioxidants detoxifying accumulated ROS, was observed in algae exposed to oxidative stress (Butow *et al*., 1994; Malanga and Puntarulo, 1995; Malanga *et al*., 1997; Collén and Davison, 1999*b*; Aguilera *et al*., 2002; Rijstenbil, 2002). It is known that the antioxidant defence mechanism against ROS is pivotal for algal survival under stressful conditions; higher antioxidant contents and antioxidant enzyme activities are associated with higher stress tolerance in algae (Butow *et al*., 1994; Collén and Davison, 1999*a*, *b*). The comparison of antioxidant enzyme activity among Arctic marine macroalgae to UV radiation showed that algal tolerance to oxidative stress was correlated with high SOD, APX, and CAT activities (Aguilera *et al*., 2002).

As far as is known, the mechanisms in the regulation of the antioxidant defence system in marine macroalgae under UV-B stress are still under debate. This study has been conducted to examine the regulation of the antioxidant defence system by UV-B in the tropical chlorophyte *Ulva fasciata* Delile by exposure of thallus segments (1 cm in length) to varying UV-B doses. The growth rate and the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) as cell viability (Chang *et al*., 1999) were determined as the parameters to elucidate the UV-B tolerance limit. The extent of lipid peroxidation estimated by a thiobarbituric acid reacting substance (TBARS) and the contents of peroxide served to indicate the degree of oxidative damage. The contents of both thallus and seawater H_2O_2 were quantified as the representatives of ROS generation by *U. fasciata*. The dynamics of water-soluble antioxidants and ROS detoxifying enzyme activities were assessed. An attempt was also made to investigate whether $O_2^{\cdot-}$ and H_2O_2 are involved in the UV-B regulation of the enzymatic defence system in *U. fasciata* by examining the responses of thallus and seawater H_2O_2 production, the levels of lipid peroxidation (TBARS), the contents of water-soluble antioxidants, and the activities of H_2O_2 scavenging enzymes to a general free radical scavenger, sodium benzoate (SB) (Hung and Kao, 2004), a specific $O_2^{\cdot-}$ scavenger, 1,2-dihydroxy-benzene-3,5-disulphonic acid (Tiron) (Wise and Naylor, 1987), or a H_2O_2 scavenger, dimethylthiourea (DMTU) (Levine *et al*., 1994).

Materials and methods

Algal materials, treatments and seawater samples

Ulva fasciata at 15–25 cm plant height was collected between August and November 2004 from the high intertidal regions at Hsitzu Bay, Kaohsiung, Taiwan. After harvesting, whole algae were extensively washed with natural seawater to remove any attached sand and the rhizoidal portions were removed to avoid microbial contamination in the following culture. Thalli were preincubated at 25 °C for 14 d in 35‰ nutrient-enriched artificial seawater (ASW) containing 403.5 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 30 mM $MgSO_4$, 10 mM TRIS–HCl (pH 8.0), N- (NH_4^+ and NO_3^-) and P- (PO_4^{3-}) free Provasoli nutrient solutions (Provasoli, 1968), 3 mM $NaHCO_3$, 400 μM

$NaNO_3$, and 20 μM Na_2HPO_4. The photoperiod was 12 h and the photosynthetically active radiation (PAR, 400–700 nm) was 100–120 μmol photons m^{-2} s^{-1} in the absence of algae, achieved by 20 W cool-fluorescent lamps (FL20D, China Electric Apparatus Ltd., Taiwan, ROC). Before UV-B treatment, thallus segments 1 cm long were excised and approximately 30 g wet weight (w. wt.) of thallus segments were first incubated in a Petri dish (internal diameter: 30 cm) containing 2.0 l of 35‰ nutrient-enriched ASW for a 24 h incubation under light condition (150 μmol photons m^{-2} s^{-1} PAR provided by 40 W cool-fluorescent lamps). After incubation, healthy thallus segments of 0.7 g w. wt. without bleaching were transferred to smaller Petri dishes (internal diameter: 10 cm) containing 30 ml of 35‰ nutrient-enriched ASW before being treated with varying UV-B doses (280–320 nm) (0, 0.5, 1, 2.5, 5, 10, and 20 W m^{-2}), by the exposure of thallus segments to UV-B lamps (TLD 15W/08, UVP, Japan) covered with 10 sheets of cellulose acetate film (to cut off UV-C radiation below 290 nm) 35 μm thick (Electron Microscopy Sciences, Hatfield, PA, USA) under 150 μmol photons m^{-2} s^{-1} PAR provided by 40 W cool-fluorescent lamps (FL40D, China Electric Apparatus Ltd., Taiwan). UV-B was only provided in the light and was turned off in the dark period. The photoperiod was 12 h. Because the average UV-B dosage at the habitat of this chlorophyte (high intertidal regions at Hsitzu Bay, Kaohsiung, Taiwan) ranged from 1.23±0.35 (mean ±SD, *n*=87) (January–April 2004) to 3.65±0.28 (mean ±SD, *n*=115) (June–September 2004) W m^{-2} according to the data determined 50 cm underwater between 10.00 h and 14.00 h using UV meters (RM11, Dr Grobel, UV-Electronic, GmbH), the UV-B irradiance used in this study was set at 0, 0.5, 1, 2.5, 5, 10, and 20 W m^{-2}. The data recorded in 2004 showed that the maximum summer Taiwan exposure of 4.08±0.53 W m^{-2} occurred between 13.00 h and 14.00 h on 14 July of 2004. The biologically weighted UV-B dosages over 12 h according to the generalized plant action spectrum (normalized to 300 nm; Caldwell, 1971) for the low-, medium-, and high-UV-B were 0.5–1 W m^{-2} (22–43 kJ m^{-2} d^{-1}), 2.5–5 W m^{-2} (65–108 kJ m^{-2} d^{-1}), and 10–20 W m^{-2} (432–864 kJ m^{-2} d^{-1}), respectively. ASW media were changed every day.

To elucidate the involvement of H_2O_2 in the UV-B-induced antioxidant defence system, thallus segments (0.7 g w. wt.) after incubation as mentioned above were first cultured in a Petri dish (internal diameter: 10 cm) containing 30 ml of 10 mM SB, 100 μM Tiron, or 100 μM DTMU (prepared in 35‰ nutrient-enriched ASW) for 1 h in darkness and then transferred to 2.5 W m^{-2} UV-B radiation for 12 h under 150 μmol photons m^{-2} s^{-1} PAR provided by 40 W cool-fluorescent lamps (FL40D, China Electric Apparatus Ltd., Taiwan, Republic of China).

After treatments, thallus segments were sampled and divided into three parts: the first part was immediately used for the TTC reduction ability assay, the second part for the determination of lipid peroxidation and the contents of peroxide, H_2O_2 and water-soluble antioxidants was fixed in liquid nitrogen and kept in a freezer at −20 °C until analysis, and the third part was fixed in liquid nitrogen and lyophilized at −40 °C for the enzyme assay.

For the determination of seawater H_2O_2 and peroxide contents, seawater samples were collected daily and assayed immediately. In this study, each Petri dish was considered as a replicate in all the statistical analysis and there were three replicates for each treatment.

Determination of TTC reduction ability

To determine the cellular activity, freshly sampled thallus segments of approximately 0.05 g w. wt. were incubated at 25 °C in 1.5 ml of 50 mM potassium phosphate buffer (pH 7.4) containing 0.8% (w/v) 2,3,5-triphenyltetrazolium chloride (TTC) and 35‰ ASW under darkness for 16 h (Chang *et al*., 1999). After washing three times with 10 ml of 35‰ ASW, intracellular insoluble formanzan was extracted twice with 5 ml of 95% ethanol at 80 °C for 20 min and the ethanol extracts were combined. After making up to 10 ml, the absorbance of combined ethanol extract was determined at A_{530}. The A_{530} values of UV-B-treated thallus segments were calculated as the percentage of the control, that is, the UV-B-free treatment.

Determination of chlorophyll a *and carotenoid contents*

Thallus segments of approximately 0.02 g w. wt. were ground in liquid nitrogen and then pigments were extracted using 2 ml of chilled 80% acetone at 4 °C for 10 min in darkness by shaking at a rate of 100 rpm. After centrifugation at 12 000 *g* at 4 °C for 5 min, the supernatant was collected and the volume was adjusted to 2 ml with 80% acetone. The absorbance of the supernatant was detected at 480, 645, and 663 nm for the estimation of chlorophyll *a* and carotenoid (β-carotene) contents. The content of chlorophyll *a* was estimated by the equation:

$$\text{chl } a \ (\mu g \ \text{ml}^{-1}) = 12.7(A_{663}) - 2.69(A_{645})$$

(Arnon, 1949), and the absorbance of carotenoid in the presence of chlorophylls was estimated by the equation:

$$A_{car} = A_{480} + 0.114(A_{663}) - 0.638(A_{645})$$

(Grobe and Murphy, 1998) and then A_{car} was converted to carotenoid contents by the extinction coefficient of 28 000 mg^{-1} cm^{-1}. The extinction coefficient of chlorophyll *a* and β-carotene was checked by chlorophyll *a* (from *Anacystis nidulans*, Product code: C6144, Sigma, St Louis, MI, USA) and β-carotene (from carrots, Product code: C4646, Sigma, St Louis, MI, USA) purchased from Sigma. The contents of chlorophyll *a* and carotenoid were expressed as nmol g^{-1} w. wt based on the molecular weight of 893.5 and 536.9 g mol^{-1} for chlorophyll *a* and β-carotene, respectively.

Determination of lipid peroxidation, peroxide and H_2O_2 *contents*

Thallus segments of approximately 0.1 g w. wt. were ground in liquid nitrogen and then 1 ml of 5% (w/v) trichloroacetic acid (TCA) was added. After centrifugation at 12 000 *g* for 10 min at 4 °C, the supernatant was collected as a TCA extract for the determination of lipid peroxidation, peroxide, and H_2O_2.

The levels of lipid peroxidation were determined from the thiobarbituric acid reacting substance (TBARS) contents resulting from the thiobarbituric acid (TBA) reaction as described by Health and Packer (1968). The TBARS contents were calculated based on A_{532}–A_{600} with the extinction coefficient of 155 mM^{-1} cm^{-1}.

Thallus and seawater peroxide contents were determined by the absorbance at A_{480} and extinction coefficient of 13.93 μM^{-1} cm^{-1} according to Sagisaka (1976). Because seawater was changed every day, the contents of peroxide in seawater were determined every day and they were summed as cumulative seawater peroxide over 4 d.

Thallus H_2O_2 contents were determined based on the decomposition of H_2O_2 by peroxidase as described by Okuda *et al*. (1991). KOH (4 M) of 11.51 μl was added to 0.2 ml supernatant to adjust the pH to 7.5 and the mixture was centrifuged at 12 000 *g* for 1 min at 4 °C. The supernatant was collected and applied to a 1 ml column of Amberlite IRA-410, and residual H_2O_2 was washed out by 800 μl of distilled water. The contents of H_2O_2 in the eluate were determined within 10 min. For the determination of H_2O_2 in the eluate, 0.4 ml of 12.5 mM 3-dimethylaminobenzoic acid (DMAB), 0.4 ml of 10 mM 3-methyl-2-benzothiazoline hydrazone (MBTH), and finally 0.02 ml of 0.25 unit ml^{-1} horsedish peroxidase (Sigma, USA) was added and then the reaction was measured at 590 nm for 3 min. The amount of thallus H_2O_2 was estimated from the H_2O_2 standard curve that had been determined as described above.

Seawater H_2O_2 contents were also determined following the same method for thallus H_2O_2 determination. One ml of the sampled

seawater was immediately passed through a 1 ml column of Amberlite IRA-410 and then the residual H_2O_2 in the column was eluted by applying 800 µl Milli-Q water. Eluates were combined and adjusted to pH 7.0 with 4 M KOH. Seawater H_2O_2 contents were calculated based on the calculation from a series of H_2O_2 concentrations prepared in nutrient-enriched ASW. H_2O_2 contents in seawater were determined daily and they were summed as cumulative seawater H_2O_2 over 4 d.

Determination of ascorbate and glutathione contents

Thallus segments of approximately 0.1 g w. wt. were ground in liquid nitrogen and then 1 ml of 5% (w/v) trichloroacetic acid (TCA) was added. After centrifugation at 12 000 *g* for 10 min at 4 °C, the supernatant was collected as a TCA extract for the determination of water-soluble antioxidant contents.

The measurement of total ascorbate and reduced ascorbate (AsA) contents were modified from the method of Hodges *et al*. (1996). Total ascorbate contents were determined in a 1 ml mixture containing 200 µl TCA extract, 50 mM potassium phosphate buffer (pH 7.4), 3 mM EDTA, and 1 mM dithiothreitol (DTT). The mixture was incubated at 25 °C for 10 min, 100 µl of *N*-ethylmaleimide was added, and then 400 µl of 0.61 M TCA, 400 µl of 0.8 M orthophosphoric acid, and 400 µl of α,α′-bipyridyl were added. Finally, 200 µl of $FeCl_3$ was added and the mixture was incubated in a 55 °C water bath for 10 min and the absorbance was detected at A_{525}. AsA contents were determined by adding distilled water instead of DTT and *N*-ethylmaleimide and then followed the same method as above. Total AsA and AsA contents were estimated from the standard curve of 0–40 nmol L-AsA determined by the above methods. DHA contents were calculated by the subtraction of AsA from total AsA.

Total glutathione contents were determined by the absorbance at 570 nm according to the method of Griffiths (1980). The contents of glutathione (reduced form) were estimated from the standard curve of 0–20 nmol glutathione. After the removal of glutathione by 2-vinylpyridine derivatization, glutathione disulphide contents were determined, and the glutathione contents were calculated by the subtraction of glutathione disulphide contents from total glutathione contents.

Determination of antioxidant enzyme activity

Lyophilized thallus discs of approximately 0.005 g dry wt. (d. wt.) were first homogenized in liquid nitrogen and 0.6 ml of 0.1 M sodium phosphate buffer (pH 6.8) containing 1% (w/v) polyvinylpolypyrrolidone (PVPP) and 0.25% (v/v) Triton X-100 was then added. After centrifugation at 12 000 *g* for 10 min at 4 °C, the supernatant was used for enzyme activity assay of SOD, CAT, POX, GR, MDHAR, and DHAR. For the determination of APX, 0.005 g d. wt. of lyophilized thallus discs was homogenized in liquid nitrogen and 0.6 ml of extraction buffer (0.1 M sodium phosphate buffer (pH 6.8) containing 1% (w/v) PVPP, 0.25% (v/v) Triton X-100, and 0.5 mM L-ascorbate) was added. After centrifugation at 12 000 *g* for 10 min at 4 °C, the supernatant was used for the APX assay. The soluble protein contents were determined by the Coomassie blue dye binding method (Bradford, 1976) with bovine serum albumin as the standard curve.

The CAT activity was measured at A_{420} for H_2O_2 decomposition rate using the extinction coefficient of 40 mM^{-1} cm^{-1} according to Kato and Shimizu (1987). Guaiacol POX activity was determined by the formation rate of tetraguaiacol detected at A_{470} with the extinction coefficient of 26.6 mM^{-1} cm^{-1} according to Kato and Shimizu (1987). SOD activity was determined by the inhibition of photochemical inhibition of nitro blue tetrazolium according to Giannopolitis and Ries (1977). APX activity was determined at A_{290} for DHA according to the extinction coefficient of 2.8 mM^{-1} cm^{-1} (Nakano and Asada, 1981). GR activity was monitored by A_{340} for β-NADPH oxidization as GSSG reduction according to Schaedle and Bassham (1977).

Both MDHAR and DHAR activities were determined according to Hou and Lin (1997). DHAR activity was measured by monitoring the absorbance at 265 nm for 3 min in the reaction mixture that consisted of enzyme extract, 50 mM Na-phosphate buffer (pH 7.0), 0.3 mM glutathione, 0.06 mM Na_2EDTA and 0.2 mM DHA. MDHAR activity was measured by the monitoring the absorbance at 340 nm for 3 min in 50 mM Na-phosphate buffer (pH 7.6), 0.1 mM β-NADPH and 0.1 unit AsA oxidase (Sigma, MO, USA) and 2.5 mM AsA. Non-enzymatic reduction of DHA or MDHA in phosphate buffer was measured in a separate cuvette at the same time.

Chemicals and statistical analysis

Chemicals were purchased from Merck (Germany) or Sigma (USA). The data were analysed by SAS (SAS version 8.1, NC, USA). The present results were the mean of three replicates with a Petri dish as a replicate. The effects of UV-B dose on TTC reduction ability, TBARS contents, peroxide contents, H_2O_2 contents, water-soluble antioxidant contents, and enzyme activities, and the effects of ROS scavengers were analysed by 1-way analysis of variance (ANOVA). The difference among means was analysed by Duncan's new multiple range test following by significant ANOVA at $P < 0.05$.

Results

Growth rate, TTC reduction ability, and the contents of chlorophyll a*, carotenoid, TBARS, peroxide, and* H_2O_2

The tolerance limit of *U. fasciata* to UV-B exposure was evaluated by the changes of growth rate, TTC reduction ability, and chlorophyll *a* and carotenoid contents 4 d after exposure to varying UV-B doses. The data in Fig. 1 show that growth rate, TTC reduction ability, chlorophyll *a* contents, and carotenoid contents were all affected by UV-B (1-way ANOVA, $P < 0.05$). Growth rate (Fig. 1A) and TTC reduction ability (Fig. 1B) were not affected by UV-B ranging from 0.5–5 W m^{-2} and were significantly decreased by UV-B ≥10 W m^{-2}. Chlorophyll *a* (Fig. 1C) and carotenoid (Fig. 1D) contents were not influenced by the low UV-B dose ranging from 0.5–1 W m^{-2} and slightly declined at 2.5–5 W m^{-2} UV-B followed by a marked drop at 10–20 W m^{-2} UV-B. The changes in lipid peroxidation and total peroxide production were in contrast to pigments. Compared with the control, TBARS contents remained unchanged on exposure to UV-B ranging from 0.5–1 W m^{-2} and then slightly rose at 2.5–5 W m^{-2} UV-B followed by a marked increase at 10–20 W m^{-2} (Fig. 2A). Both thallus total peroxide contents (day 4) (Fig. 2B) and cumulative seawater peroxide contents (over 4 d) (Fig. 2C) were not affected by 0.5–1 W m^{-2} UV-B followed by a slight increase at 2.5–5 W m^{-2} and a marked increase at 10–20 W m^{-2}.

Next, the effects of UV-B on H_2O_2 production by thallus segments was investigated. The preliminary experiments found a large quantity of H_2O_2 detected in seawater after incubation of *U. fasciata* thallus segments. It seems to suggest that H_2O_2 produced by thallus segments could be excreted into seawater. Therefore, in addition to H_2O_2 in thallus segments, the changes in H_2O_2 contents in seawater

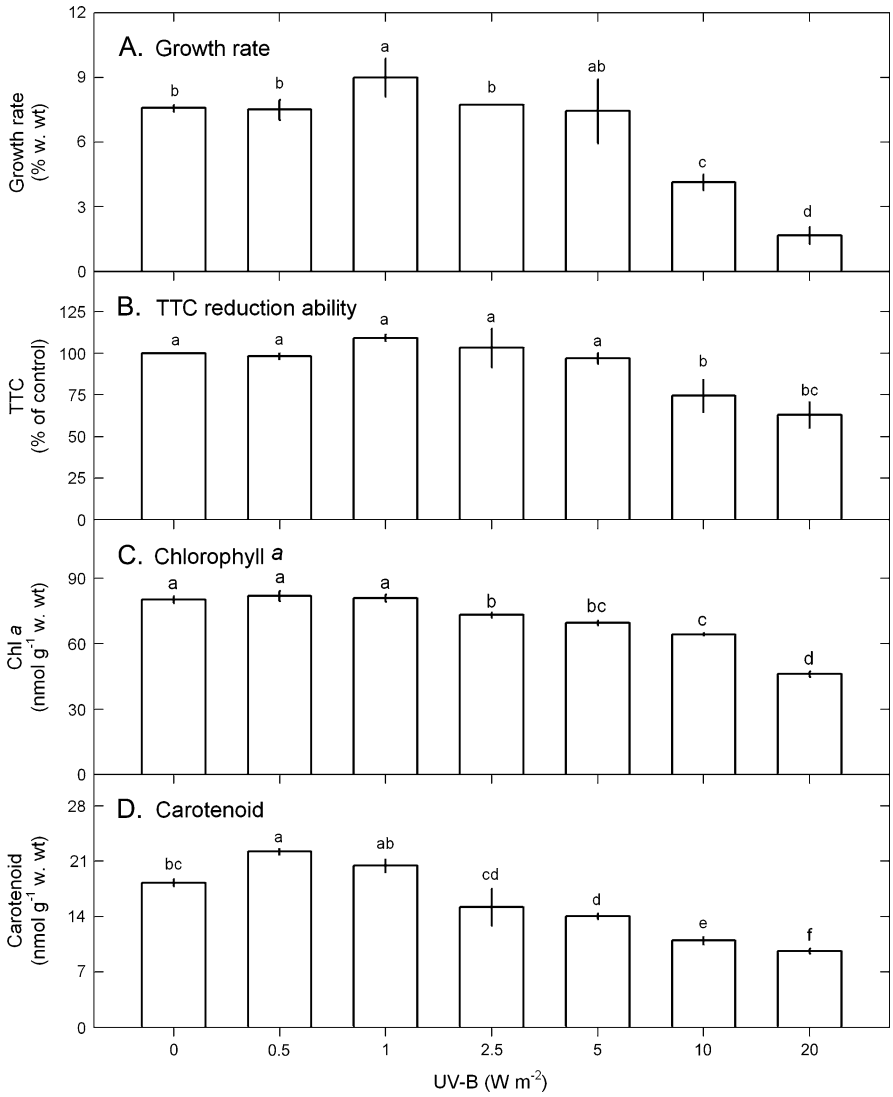

Fig. 1. Changes in growth rate (A), TTC reduction ability (B), chlorophyll *a* (C), and carotenoid (D) in *Ulva fasciata* in response to varying UV-B doses. Data are present as means ±SD (n=3) and different letters indicate significant difference among treatments.

were also quantified. The data in Fig. 2 show that thallus H_2O_2 contents (day 4) and cumulative seawater H_2O_2 contents (over 4 d) were affected by UV-B (1-way ANOVA, P <0.05). Compared with the control, thallus H_2O_2 contents were not affected by UV-B ranging from 0.5–1 W m^{-2} and then slightly rose at 2.5–5 W m^{-2} UV-B followed by a significant increase at 10–20 W m^{-2} (Fig. 2D). Cumulative seawater H_2O_2 contents over 4 d showed a similar pattern (Fig. 2E). The amount of thallus H_2O_2 contents at day 4 and cumulative seawater H_2O_2 contents over 4 d appears to be a dose-response, indicating that the generation of H_2O_2 by thallus segments is positive depending on UV-B dose. In addition, the higher H_2O_2 contents in seawater than those in the thallus segments reflect that most of H_2O_2 produced by thallus segments could be excreted into seawater, and it was found that the release of H_2O_2 was increased by increasing UV-B radiation. Thus, the defence system of *U. fasciata* is working against UV-B-induced H_2O_2 accumulation and oxidative stress that can be alleviated at 0.5–1 W m^{-2} but could not be overcome when UV-B was ⩾2.5 W m^{-2}, thus leading to oxidative damage, especially at 10–20 W m^{-2}.

Ascorbate and glutathione contents

Ascorbate and glutathione contents were determined to test whether the water-soluble antioxidants are involved in the defence system against UV-B-induced oxidative stress in *U. fasciata*. The contents of total AsA, AsA, and DHA and the ratios of AsA/DHA were significantly influenced by UV-B (ANOVA, P <0.05) (Fig. 3). Total AsA (Fig. 3A) and AsA (Fig. 3B) contents increased with increasing UV-B (0.5–20 W m^{-2}) while DHA contents (Fig. 3C) increased when UV-B was ⩾2.5 W m^{-2}. To explore the regeneration

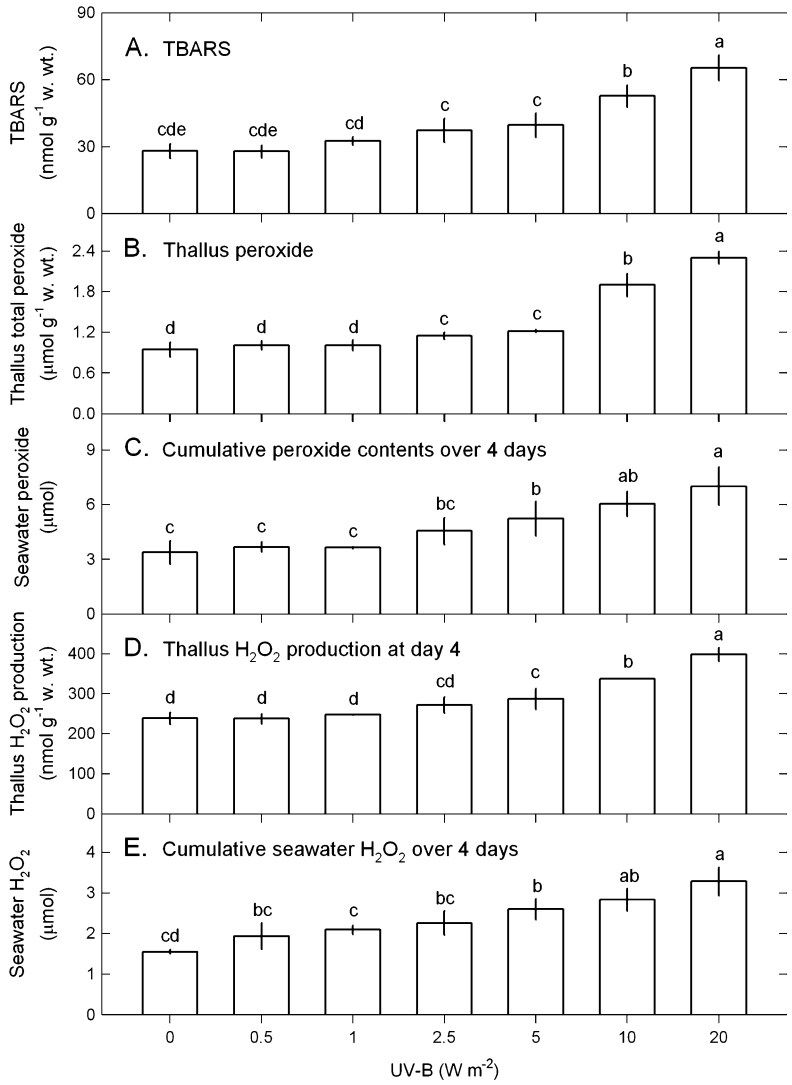

Fig. 2. Changes in the contents of TBARS (A), total peroxide in thallus (B) and seawater (C), and H_2O_2 in thallus (D) and seawater (E) in *Ulva fasciata* in response to varying UV-B doses. Data are present as means ±SD (n=3) and different letters indicate significant difference among treatments.

rate of AsA, the ratio of AsA/DHA was calculated. The data in Fig. 3D show that, upon exposure to UV-B, the ratio of AsA/DHA increased with the maximum occurring in a range from 0.5–2.5 W m^{-2} followed by a drop when UV-B was ⩾5 W m^{-2}.

Total glutathione, glutathione, and glutathione disulphide contents and glutathione/glutathione disulphide ratios were affected by UV-B (ANOVA, P <0.05) (Fig. 4). The contents of total glutathione (Fig. 4A), glutathione (Fig. 4B), and glutathione disulphide (Fig. 4C) were increased with increasing UV-B dose, with the maximum occurring in a range of 5–10 W m^{-2} followed by a drop at 20 W m^{-2}. Glutathione/glutathione disulphide ratio as an indicator of glutathione regeneration rate decreased with increasing UV-B dose because the magnitude of glutathione disulphide increments was higher than that of glutathione increments (Fig. 4D).

Enzyme activities

UV-B significantly affected SOD activity (ANOVA, P <0.05) that increased as UV-B increased from 0.5–2.5 W m^{-2} and subsequently decreased upon exposure to a higher UV-B dose, that is, 10–20 W m^{-2} (Fig. 5A).

Both CAT and POX activities were affected by UV-B (P <0.05); the CAT activity increased as UV-B increased from 0.5–2.5 W m^{-2} and then decreased to the level lower than the control when UV-B was ⩾5 W m^{-2} (Fig. 5B), while the POX activity only increased at 0.5 W m^{-2} (Fig. 5C).

The activities of enzymes in the ascorbate–glutathione cycle were affected by UV-B (P <0.05) (Fig. 5). Upon exposure to UV-B, both APX (Fig. 5D) and GR (Fig. 5E) activities increased fast with the maximum occurring at 0.5–1 W m^{-2} and then decreased gradually as UV-B increased from 2.5 to 20 W m^{-2}, but they were still higher than the control. MDHAR activity increased when UV-B

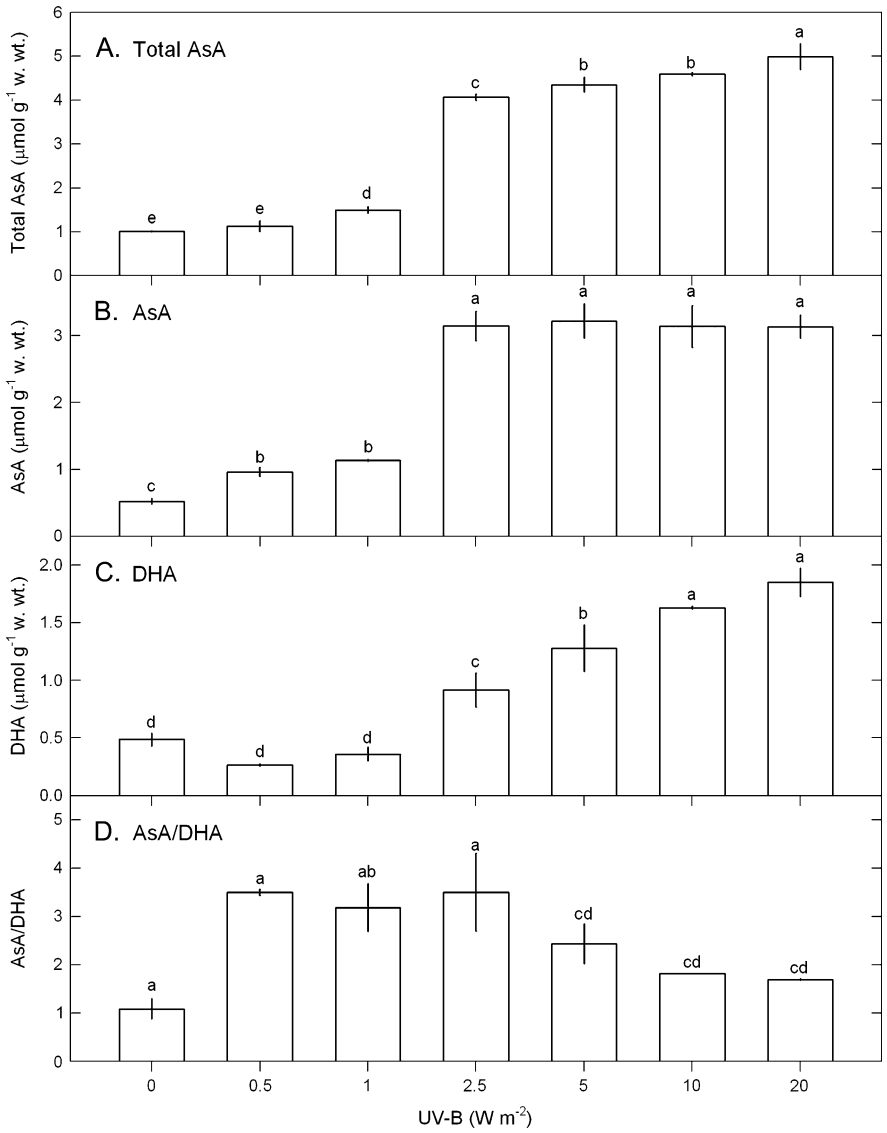

Fig. 3. The contents of total AsA (A), AsA (B), DHA (C), and the ratios of AsA/DHA (D), in *Ulva fasciata* in response to varying UV-B doses. Data are present as means ±SD (n=3) and different letters indicate significant difference among treatments.

was ⩾2.5 W m^{-2}; the maximum occurring at 5 W m^{-2} and then a decrease at 10–20 W m^{-2}, they were still higher than the control (Fig. 5F). Compared with the response of MDHAR under high UV-B doses (2.5–20 W m^{-2} UV-B), DHAR responded to low UV-B doses ranging from 0.5–5 W m^{-2} with the maximum at 1 W m^{-2} (Fig. 5G).

Effects of SB, Tiron, and DMTU on UV-B-induced oxidative stress, antioxidant contents, and enzyme activities

The short-term exposure to 2.5 W m^{-2} UV-B for 12 h increased the contents of TBARS and thallus H_2O_2 (Fig. 6), the activities of SOD, CAT, APX, GR, and DHAR (Fig. 7), and the contents of AsA and glutathione. However, the activities of POX and MDHAR were not induced by this short-term UV-B exposure.

The UV-B-induced increase in the contents of TBARS and thallus H_2O_2 was inhibited by SB, Tiron, and DMTU (Fig. 6). Increased SOD, CAT, APX, GR, and DHAR activities by UV-B exposure were also depressed by SB, Tiron, and DMTU (Fig. 7). However, POX and MDHAR activities were not affected (Fig. 7). The UV-B-induced increase in total AsA, AsA, and DHA contents and AsA/DHA ratios were inhibited by SB, Tiron, and DMTU (Fig. 8). The UV-B-induced increase in the contents of total glutathione, glutathione, and glutathione disulphide was also inhibited by SB, Tiron, and DMTU, and the decrease in the ratios of glutathione/glutathione disulphide in UV-B-treated thalli was reversed by SB, Tiron, and DMTU (Fig. 8).

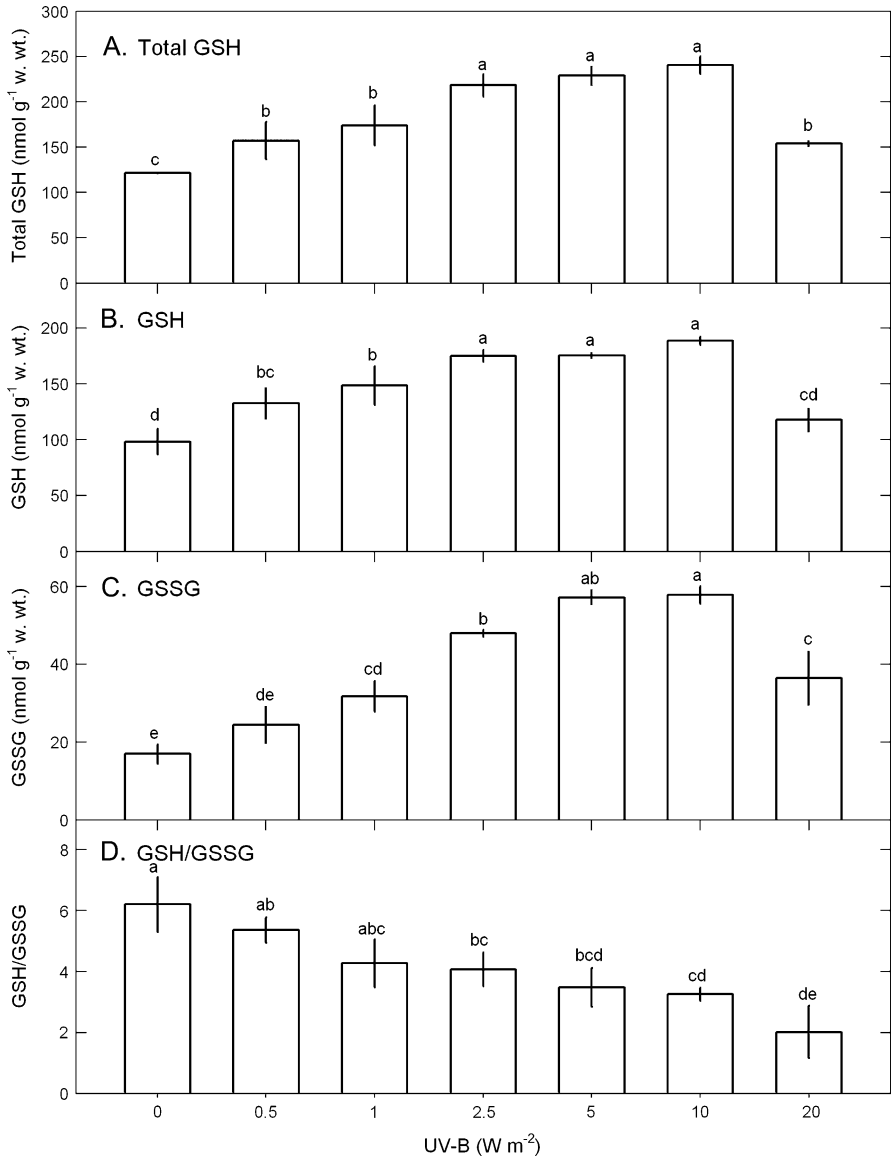

Fig. 4. The contents of total glutathione (A), glutathione (B), glutathione disulphide (C), and the ratios of glutathione/glutathione disulphide (D) in *Ulva fasciata* in response to varying UV-B doses. Data are present as means ±SD (n=3) and different letters indicate significant difference among treatments.

Discussion

This study was aimed at a better understanding of the regulation of the antioxidant defence system in a tropical intertidal chlorophyte *U. fasciata* in response to varying UV-B doses. Using growth rate, TTC reduction ability, photosynthesis pigments, total peroxide, and lipid peroxidation as indicators, the UV-B tolerance limit in *U. fasciata* has been identified. Increased TBARS and total peroxide contents but decreased chlorophyll *a* and carotenoid contents in medium (2.5–5 W m^{-2}) and high (10–20 W m^{-2}) UV-B doses suggest the occurrence of oxidative damage when UV-B was ⩾2.5 W m^{-2}. However, the oxidative damage was mild in medium UV-B doses as indicated by a relatively small increase in lipid peroxidation and total peroxide production and a slight decrease in the chlorophyll *a* and carotenoid contents. In addition, both growth rate and TTC reduction ability were only decreased by high UV-B doses. This means that the defence and repairing systems are operating normally in low (0.5–1 W m^{-2}) and medium UV-B radiation doses with 5 W m^{-2} as the tolerance limit. The limit of *U. fasciata* to UV-B radiation is similar to the current maximum summer Taiwan exposure (4.08± 0.53 W m^{-2}). It is reasonable for this high intertidal chlorophyte, in which significant UV-B exposure will occur when the alga is submerged. Ecophysiological studies have suggested that the physiological responses of macroalgae to UV-B are associated with their zonation pattern; for

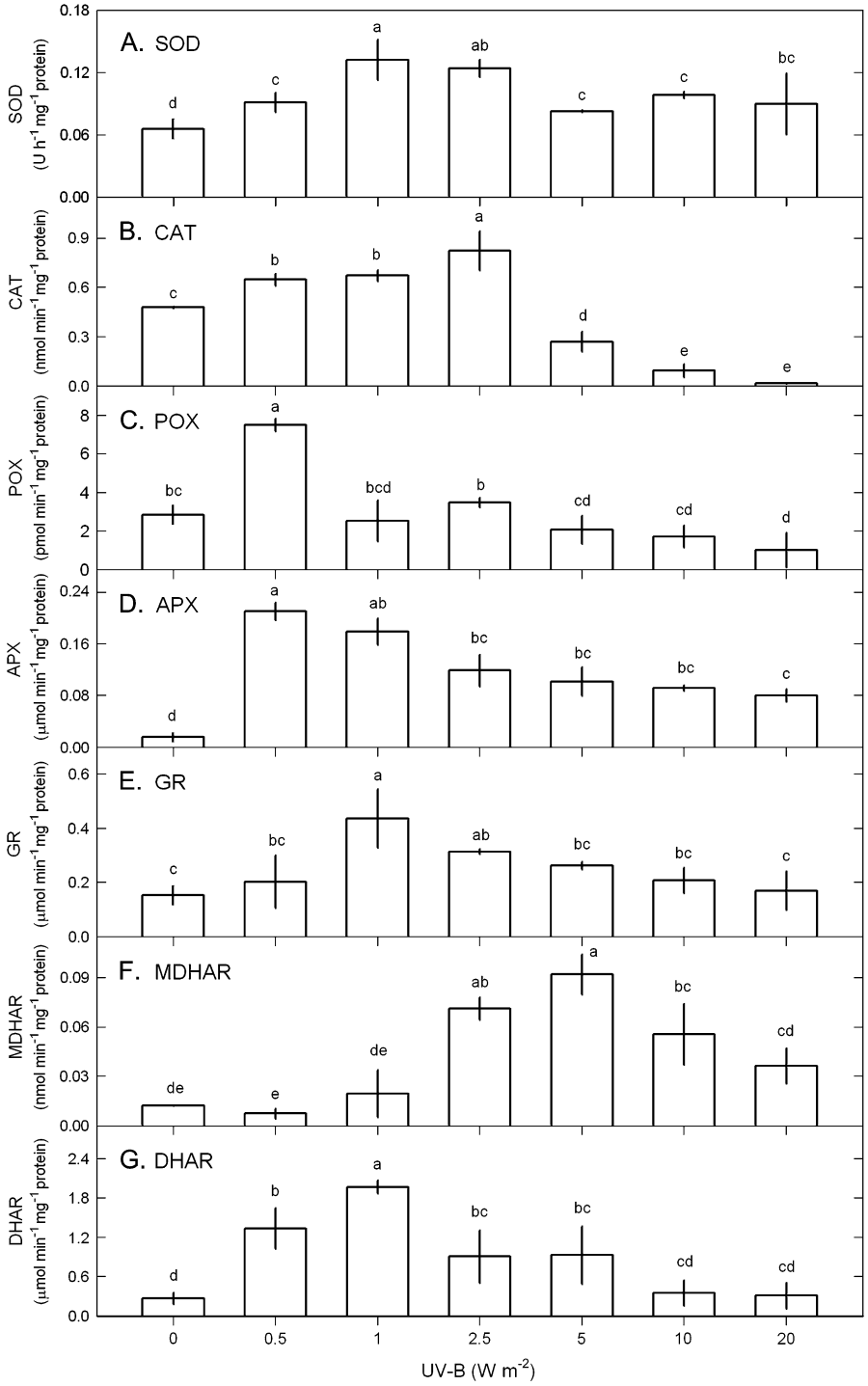

Fig. 5. The activities of SOD (A), CAT (B), POX (C), APX (D), GR (E), MDHAR (F), and DHAR (G) in *Ulva fasciata* in response to varying UV-B doses. Data are present as means ±SD (n=3) and different letters indicate significant difference among treatments.

example, the sensitivity of photosynthetic electron transport activity to UV-B is correlated with the vertical distribution pattern of the alga (Dring *et al*., 1996; Bischof *et al*., 1998). A survey of the UV tolerance of marine rhodophytes from tropical habitats showed that UV exposure caused less pronounced effects in intertidally occurring species than in subtidal species (van de Poll *et al*., 2003). Arctic marine macroalgal species from eulittoral and upper sublittoral regions also showed higher antioxidant enzyme activities than species from lower sublittoral regions upon exposure to

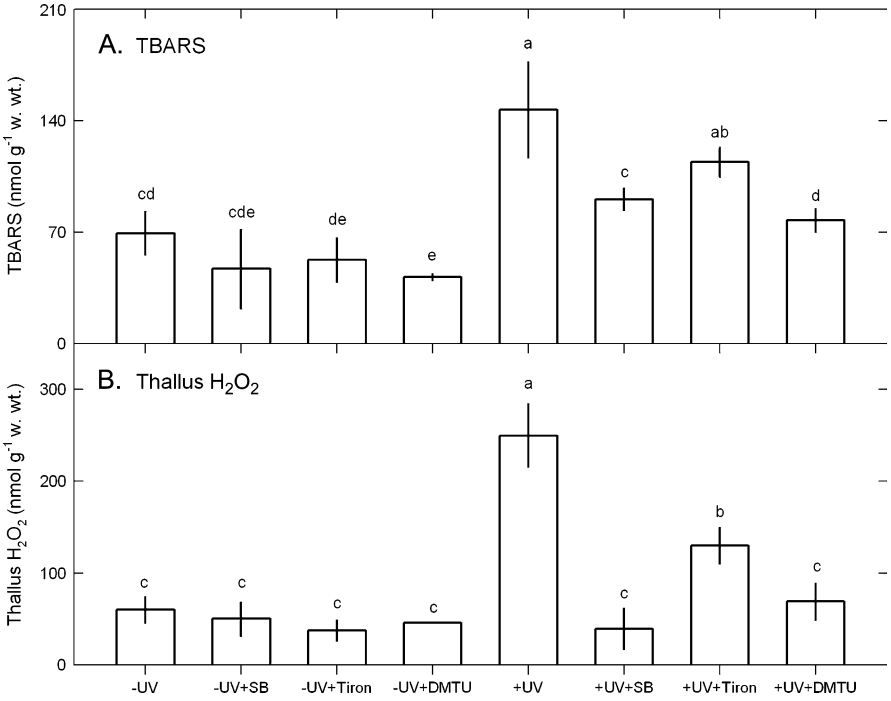

Fig. 6. Effects of 10 mM SB, 100 μM Tiron, or 100 μM DTMU on the contents of TBARS (A) and thallus H_2O_2 (B) in *Ulva fasciata* on exposure to UV-B (2.5 W m^{-2}). Data are present as means ±SD (n=3) and different letters indicate significant difference among treatments.

UV (Aguilera *et al*., 2002). It would be expected that the antioxidant defence system has evolved to protect high intertidal *U. fasciata* from UV-B-induced oxidative stress.

Increasing H_2O_2 contents in thallus segments and seawater with increasing UV-B doses reflect the stimulation of H_2O_2 generation in *U. fasciata* by UV-B. Although the contents of $O_2^{\cdot-}$ have not been determined in this paper, the inhibition of UV-B (2.5 W m^{-2})-induced H_2O_2 generation by Tiron, a specific $O_2^{\cdot-}$ scavenger, indicates that the first ROS species generated by *U. fasciata* under UV-B stress is $O_2^{\cdot-}$ which is the primary source of H_2O_2. Because $O_2^{\cdot-}$ generated in *Ulva* spp. exposed to UV-B could be dismutated to H_2O_2 by SOD (Bischof *et al*., 2000), the SOD-mediated dismutation of $O_2^{\cdot-}$ to H_2O_2 may contribute to H_2O_2 production in *U. fasciata* under UV-B radiation. However, SOD activity of *U. fasciata* upon exposure to elevated UV-B flux increased with a peak at 2.5 W m^{-2} followed by a decrease at higher UV-B doses, reflecting that, in addition to SOD-mediated $O_2^{\cdot-}$ dismutation, H_2O_2 could be generated from other origins, for example, pH-dependent cell wall peroxidase, amine oxidase, and germin-like oxalate oxidase (Wojtaszek, 1997). Overall, the present study shows that $O_2^{\cdot-}$ and H_2O_2 are the ROS species generated by *U. fasciata* following UV-B exposure.

The excessive production of $O_2^{\cdot-}$ and H_2O_2 causes oxidative stress in *U. fasciata* by exposure to UV-B. The alleviation of oxidative damage by the inhibition of UV-B (2.5 W m^{-2})-induced H_2O_2 accumulation following the treatment of a H_2O_2 scavenger, DMTU, or a general free radical scavenger, SB, indicate that increased formation of H_2O_2 has an intimate relationship with UV-B-induced oxidative damage. The magnitude of H_2O_2 increase in thallus segments and seawater is also positively correlated with the extent of lipid peroxidation and total peroxide contents. However, this relationship only appears when UV-B was ⩾2.5 W m^{-2}, suggesting that H_2O_2 can not be fully detoxified by increasing UV-B flux ⩾2.5 W m^{-2}. A peak of SOD activity in *U. fasciata* at 2.5 W m^{-2} followed by a decrease at higher UV-B doses reflects that SOD is responsible for $O_2^{\cdot-}$ detoxification in *U. fasciata* exposed to UV-B, but the ability to detoxify $O_2^{\cdot-}$ decreases when UV-B doses ranged from 5–20 W m^{-2} compared with 2.5 W m^{-2}. SOD constitutes the first line of defence against ROS in plants (Alscher *et al*., 2002), but the responses of SOD to UV-B depend on algal species and exposure time. Although the UV-B-induced SOD activity increase has been documented in a symbiotic dinoflagellate (Lesser and Shick, 1989), the long-term UV-B exposure decreased chloroplast SOD activity in *Chlorella vulgaris* (Malanga and Puntarulo, 1995). Arctic macroalgae (chlorophyte: *Monostroma arcticum*, *Acrosiphonia penicilliformis*; rhodophytes: *Coccotylus truncates*, *Phycodry rubens*, *Palmaria palmate*, and *Devaleraea ramentacea*) also showed less SOD induction by exposure to 'UV-A+UV-B', SOD activities in some species were even depressed (Aguilera *et al*., 2002). In this case, SOD activity was substantially increased in *U. fasciata* in response to long-term (4 d) low and medium UV-B flux, but it was not induced under high UV-B flux.

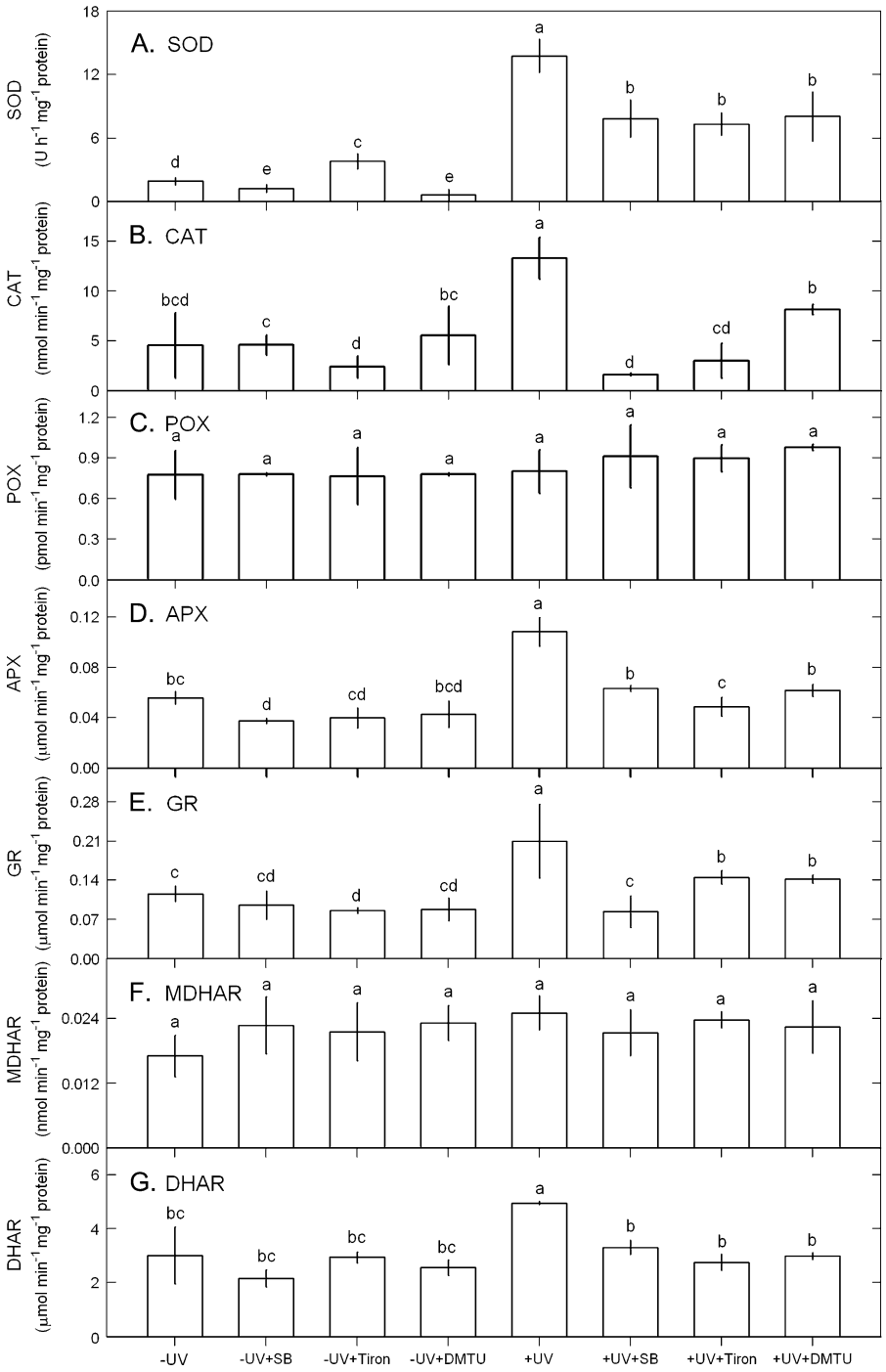

Fig. 7. Effects of 10 mM SB, 100 μM Tiron, or 100 μM DTMU on the activities of SOD (A), CAT (B), POX (C), APX (D), GR (E), DHAR (F), and MDHAR (G) in *Ulva fasciata* on exposure to UV-B (2.5 W m^{-2}). Data are present as means ±SD (n=3) and different letters indicate significant difference among treatments.

It suggests that oxidative damage in *U. fasciata* under UV-B radiation, especially high UV-B flux, is attributable to low $O_2^{\cdot-}$ detoxification ability together with high H_2O_2 accumulation.

The detection of a large quantity of H_2O_2 in seawater after incubation of *U. fasciata* thallus segments and the parallel increase of cumulative seawater H_2O_2 contents and thallus segment H_2O_2 contents in response to increasing UV-B doses seem to suggest that H_2O_2 overproduced by UV-B-treated thalli could be excreted into seawater. The ability to excrete accumulated H_2O_2 into the surrounding medium for preventing H_2O_2 toxicity has been reported in the

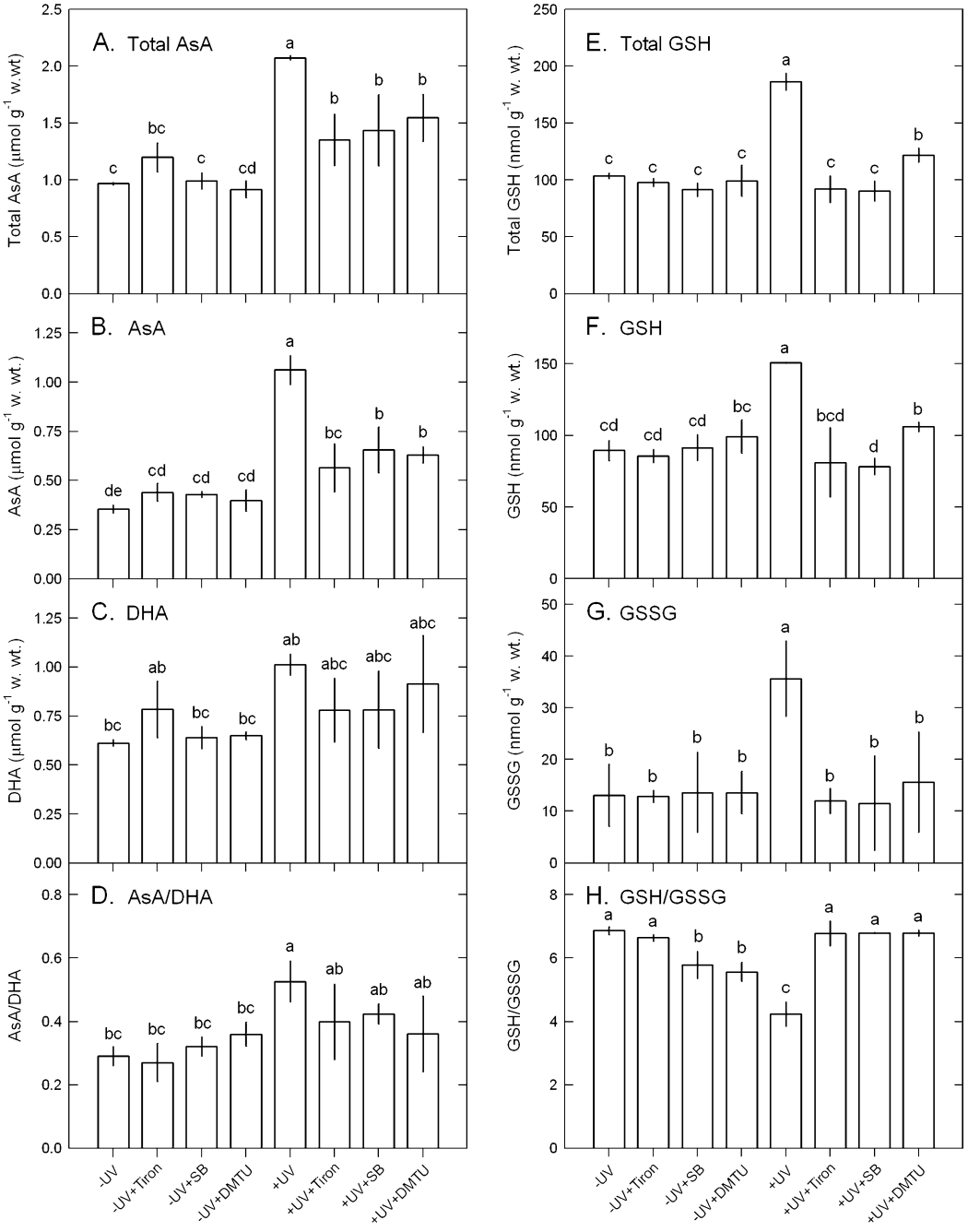

Fig. 8. Effects of 10 mM SB, 100 μM Tiron, or 100 μM DTMU on the contents of total AsA (A), AsA (B), and DHA (C), the ratios of AsA/DHA ratio (D), the contents of total glutathione (E), glutathione (F), and glutathione disulphide (G), and the ratios of glutathione/glutathione disulphide (H) in *Ulva fasciata* on exposure to UV-B (2.5 W m^{-2}). Data are present as means ±SD (n=3) and different letters indicate significant difference among treatments.

chlorophyte *Ulva rigida* C. Ag. (Collén *et al*., 1995), the brown macroalga *Fucus* sp. (Collén and Davison, 1999*a*, *b*), and the economically important carrageenophyte *Kappaphycus alvarezii* (Doty) Doty (a red macroalga) (Barros *et al*., 2003). The excretion of H_2O_2 into the surrounding water is considered one of the mechanisms for algae to avoid harmful accumulation of cellular H_2O_2 (Collén *et al*., 1995; Barros *et al*., 2003). Alternatively, H_2O_2 in the medium of UV-B-treated *U. fasciata* may be produced at the external surface of the plant cells. Studies have shown that ROS such as $O_2^{\cdot-}$ and H_2O_2 can be produced extracellularly in terrestrial plants (Bolwell, 1999), bryophytes and lichens (Minibayeva and Beckett, 2001; Beckett *et al*., 2003) through the reaction of cell wall-bound peroxidase and plasma membrane-bound NAD(P)H oxidase (Bolwell *et al*., 1998). Even if H_2O_2 was generated

externally, it is thought that thallus H_2O_2 could be diffused through the plasmalemma for releasing the toxic effects caused by harmful H_2O_2 accumulation in *U. fasciata* under UV-B stress.

Evidence shows that the availability of antioxidants and the activities of antioxidant enzymes in the ascorbate–glutathione cycle are involved in H_2O_2 scavenging in *U. fasciata* under UV-B radiation. Upon exposure to low UV-B flux (0.5–1 W m^{-2}), a significant AsA regeneration, as indicated by a marked increase in AsA/DHA ratio, suggests that a rising AsA regeneration rate is crucial for the proper scavenging of accumulated ROS in *U. fasciata* under low UV-B doses. Compared with low UV-B doses, marked increases in the contents of AsA, DHA, and total AsA, as well as high ratios of AsA/DHA in response to medium UV-B radiation (2.5–5 W m^{-2}), indicate that an increasing AsA pool in combination with the rapid regeneration of DHA to AsA are attributable to oxidative defence in response to moderate UV-B radiation. The ability of AsA regeneration is inhibited by high UV-B flux (10–20 W m^{-2}) as indicated by a significant drop in AsA/DHA ratios when UV-B increased to 10–20 W m^{-2}. Glutathione also responds to UV-B stress as one component in defence system. Similar to ascorbate, the pools of glutathione and their regeneration rates are increased for detoxifying ROS under UV-B conditions. Although glutathione/GSSG ratios were decreased by UV-B, the data that the GR activity of UV-B-treated *U. fasciata* was higher or remained unchanged compared with the control reflect that glutathione regeneration was not inhibited by UV-B, instead glutathione was consumed faster than its regeneration. The reverse of the UV-B-induced glutathione/glutathione disulphide ratio decrease by ROS scavengers (SB, Tiron, and DMTU) supports this view. In intertidal brown and red macroalgae (Collén and Davison, 1999*a*, *b*, *c*), higher antioxidant levels are positively associated with their stress tolerance. Instead of keeping a high quantity of antioxidants under normal condition, the antioxidant defence ability in *U. fasciata* is achieved by increasing the amounts of AsA and glutathione as well as their regeneration rate under UV-B stress.

Increased APX activity functions to keep the balance of cellular H_2O_2 components in *U. fasciata* in response to low UV-B flux. Both CAT and POX are also responsible for the removal of excess H_2O_2 under low UV-B flux, as indicated by increasing CAT activity with increasing UV-B radiation from 0.5–2.5 W m^{-2} and increased guaiacol POX activity at 0.5 W m^{-2}. As the substrate of APX reaction, the availability of AsA is becoming essential for *U. fasciata* under UV-B stress. Evidence shows that the recycling of AsA is mediated by MDHAR and DHAR in the generation of GR. DHAR is responsible for AsA regeneration at low UV-B doses while MDHAR operates at medium UV-B doses. Because glutathione is used as an electron donor for reducing DHA to generate AsA by DHAR and there was the same pattern of DHAR and GR upon exposure to varying UV-B doses, the decline of glutathione regeneration due to diminished GR activities when UV-B was $\geqslant$2.5 W m^{-2} explains why AsA was not regenerated via the DHAR route under medium and high UV-B flux. Although AsA and glutathione pools are increasing as the UV-B flux increases, the extent of UV-B-induced APX and GR activity increase under medium and high UV-B radiation was less than that under low UV-B flux. It is why the oxidative damage (lipid peroxidation and total peroxide production) was significant under medium and high UV-B radiation.

H_2O_2 has been known as the signal for the induction of the antioxidant defence system in plants in response to biotic and abiotic stresses (Neil *et al*., 2002*a*, *b*). Current results suggest that the induction of the antioxidant defence system in *U. fasciata* is mediated by $O_2^{\cdot-}$ and H_2O_2. The inhibition of UV-B-induced antioxidant regeneration and antioxidant enzyme activity increase by DMTU, the scavenger of H_2O_2, and Tiron, the $O_2^{\cdot-}$ scavenger, supports the idea that H_2O_2 is involved in the induction of the antioxidant defence system in *U. fasciata*. The removal of free radicals by SB, a free radical scavenger, shows a similar result. The induction of APX gene expression by H_2O_2 has also been observed in germinating rice embryos (Morita *et al*., 1999) and in *Arabidopsis* leaves (Karpinski *et al*., 1999). It seems that UV-B activates the production of $O_2^{\cdot-}$ and H_2O_2 for triggering the antioxidant defence system in *U. fasciata*.

In conclusion, the excretion of H_2O_2 as well as the availability of antioxidants and the activation of SOD, CAT, guaiacol POX, and reactive oxygen scavenging enzymes in the ascorbate–glutathione cycle serve as the defence system against oxidative stress occurring in *U. fasciata* upon exposure to UV-B. UV-B disrupts the balance between the production and removal of H_2O_2 and subsequently accumulated H_2O_2 initiates the signalling responses leading to the induction of enzymatic antioxidant defence systems to overcome ROS production in *U. fasciata*.

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